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Reticuloendotheliosis virus (REV) infection can result in immunosuppression, a runting syndrome, high mortality, acute reticulum cell neoplasia, or T-cell and/or B-cell lymphomas, in a variety of domestic and wild birds. Histopathological changes in REV infection are not sufficient to differentiate it from avian lymphoid leukosis and Marek's disease, and currently there are no available *in situ* diagnostic methods for detection of active REV presence in pathologic specimens. To develop immunohistochemistry and *in situ* hybridization assays for detection of REV active infections, experimentally inoculated Japanese quail embryos, and archived formalin-fixed paraffin-embedded tissues from natural and experimental reticuloendotheliosis cases in chickens and turkeys, were examined. The *in situ* hybridization and immunohistochemistry assays proved to be efficient for the detection of several REV strains in Japanese quail embryos during active infection, whereas these assays were much less sensitive when applied to archived tissue samples from chronically infected birds with lymphoid tumours. The diagnostic assays developed in this study have potential as diagnostic tools for detection of active REV infections.

Introduction

The viruses of the reticuloendotheliosis virus (REV) group are currently classified as gammaretroviruses and consist of pathogenic retroviruses that share structural, morphological and antigenic similarities to mammalian type C retroviruses. REVs are, however, structurally, morphologically, and immunologically unrelated to the viruses of the avian leukosis/sarcoma group of retroviruses (Purchase & Witter, 1975; Payne, 1998; Witter & Fadly, 2003). Representative strains of the REV group include the replication defective T strain and the non-defective A strain, both of which were originally isolated from turkeys (Robinson & Twiehaus, 1974; Chen *et al.*, 1987). Additional non-defective REV strains have been isolated from geese, pheasants, peafowl, Japanese quail, Greater prairie chickens (*Tympanuchus cupido pinnatus*), and Attwater's prairie chickens (*Tympanuchus cupido attwateri*). Natural or experimentally induced reticuloendotheliosis, an oncogenic and immunosuppressive dis-

ease, has also been reported in a variety of wild birds (Ley *et al.*, 1989; Hayes *et al.*, 1992; Peterson *et al.*, 2002; Witter & Fadly, 2003; Zavala *et al.*, 2006; Barbosa *et al.* 2007a,b).

The clinical outcome of REV infection ranges from a runting syndrome to acute reticulum cell neoplasia or to generation of T-cell or B-cell lymphomas that may resemble those induced by Marek's herpesvirus or avian leukosis/sarcoma viruses (Witter *et al.*, 1986; Witter & Fadly, 2003). REV also results in immunosuppression that may predispose affected birds to a variety of secondary infectious agents (Witter & Fadly, 2003). REV is widespread and may be transmitted vertically, horizontally by direct contact with infected birds, or mechanically by insects (McDougall *et al.*, 1981; Witter & Salter, 1989; Davidson & Braverman, 2005). However, most cases are due to vaccination of young commercial birds with contaminated vaccines. REV can integrate

into the genome of large DNA viruses including the viruses that cause fowlpox and Marek's disease; both of these diseases are commonly controlled by vaccination in commercial poultry flocks worldwide (Witter & Fadly, 2003). However, only those vaccine strains carrying a nearly intact REV provirus are more likely to cause disease in the field (Yuasa *et al.*, 1976; Jackson *et al.*, 1977; Isfort *et al.*, 1992; Hertig *et al.*, 1997; Singh *et al.*, 2003; Witter & Fadly, 2003).

Considering that REV can induce either T-cell or B-cell transformation, histopathology and lymphocyte markers do not allow a differential diagnosis with Marek's disease or lymphoid leukosis, which cause T-cell and B-cell transformation, respectively (Crespo *et al.*, 2002). Therefore, there are no diagnostic methods currently available for detection of active REV infection in formalin-fixed paraffin-embedded tissues (Witter & Fadly, 2003).

The aim of the present study was to develop immunohistochemical (IHC) and *in situ* hybridization (ISH) assays for detection of REV in formalin-fixed paraffin-embedded tissues of experimentally infected Japanese quail embryos and also in archived samples of tumours from REV-infected birds.

Material and Methods

Samples, viruses and inoculation. Experimentally inoculated Japanese quail embryos and archived formalin-fixed and paraffin-embedded tissues, from natural and experimental reticuloendotheliosis cases in chicken and turkey, were used in this study.

One hundred Japanese quail eggs containing 5-day-old embryos were randomly divided into five groups (*n* = 20); each group was inoculated intra-yolk with a different REV strain (Table 1). Each egg was inoculated with a viral dose of 5×10^4 tissue culture infectious units of REV. Twenty-five embryos were selected, including five representatives of each REV strain tested. Uninfected embryos (*n* = 5) were inoculated with sterile neutral phosphate-buffered saline (PBS) and used as negative controls. At 14 to 15 days of incubation, eggs were placed at 4°C as method of euthanasia.

The coelomic cavities were then opened and the whole embryos were fixed by immersion in 10% formalin for 24 h. The embryos were then transversely and serially sectioned at approximately 5 mm thickness, thus allowing histological examination of all major organs. Tissues were processed for paraffin embedding, and 4 μm sections mounted onto positively charged slides were used for ISH and IHC.

The ISH and IHC assays were also applied to archived paraffin-embedded tissues from birds either naturally or experimentally infected with REV. The 10 confirmed cases of REV infection used in this study are characterized in Table 2.

In situ **hybridization.** The protocol used in this study was modified from a previously described ISH method (Brown, 1998). Total RNA from the REV strain APC-566 was used for synthesis of the riboprobe. This REV strain is an isolate from APC, which is a genome very similar to all other published REV sequences (Zavala *et al.*, 2006; Barbosa *et al.*, 2007a,b). First-strand cDNA was synthesized using 2 μg RNA, an oligo-dT(18) primer, 0.25 mM dNTPs, and Superscript reverse transcriptase (Invitrogen, Carlsbad, California, USA), according to the manufacturer's instructions. Three pairs of primers were designed based on the REV gag genome sequence (GenBank accession number NC_006934) (Table 3). Polymerase chain reaction (PCR) amplification of fragments was performed using the following parameters: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C 30 sec, followed by a final extension step at 72°C for 7 min. PCR products were purified using a commercial PCR purification Kit (Qiagen, Valencia, California, USA), ligated into the pGEM-Teasy vector (Qiagen), and the resulting plasmids were transformed into *Escherichia coli* by heat shock. Positive colonies were identified by PCR, and then further confirmed by sequencing of both strands of the insert. Plasmid DNA was purified using a commercial mini-prep kit (Promega, Madison, Wisconsin, USA), and cleaved with *Sac*II. Linearized constructs were used for synthesis of the riboprobes by *in vitro* transcription with SP6 RNA polymerase. Three anti-sense riboprobes were generated, to create three different sizes: 500, 259 and 151 bases (Table 3). Sense riboprobes were also synthesized and used as negative controls.

Unstained slides were heated at 60°C for 20 min, and deparaffinized in Hemo-De (PMP Medical Industries, Irving, Texas, USA) for 20 min. The slides were allowed to dry at 37°C for 20 min and the sections were encircled with a hydrophobic pen. The sections were then re-hydrated in PBS plus 5 mM MgCl$_2$ for 10 min at room temperature. The slides were rinsed in 0.2 M Tris (pH 7.5) + 0.1 M glycine for 10 min at room temperature, followed by 15 min of incubation with 100 μg/ml proteinase K in 10 mM Tris (pH 7.5) with 2 mM CaCl$_2$ at 37°C. The cell preparations were washed with 0.2 M Tris (pH 7.5) with 0.1 M glycine for 2 min, followed by incubation with the pre-hybridization solution (5 × standard sodium citrate, 50% formamide, 5% modified milk protein, 1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulphate (SDS)) for 1 h at 42°C. Each slide was incubated with 2 μl riboprobe diluted in 70 μl pre-hybridization solution. The slides were then covered with siliconized slips, the edges of which were sealed with nail hardener, and incubated overnight at 4°C. Cover slips were removed, and sequential stringent washes were performed with 2 × standard sodium citrate (SCC) plus 1% SDS for 30 min at 50°C; 1 × SCC plus 0.1% SDS for 30 min at 50°C; 1 × SSC for 30 min at room temperature, 0.1 × SSC for 15 min at room temperature; and buffer 1 (100 mM Tris–HCl, pH 7.6, 150 mM NaCl) for 5 min at room temperature. The slides were then incubated with a 1:300 dilution of anti-digoxigenin-AP (Roche Diagnostics, Indianapolis, Indiana, USA) with 2% sheep serum in buffer 1 for 2 h at 37°C. Sequential washes were performed as three washes with buffer 1 for 15 min at room temperature, and buffer 3 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl$_2$) for 5 min at room temperature. The substrate/chromogen (nitroblue tetrazolium chloride; Roche) and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (Roche) was added. One drop of 5 mM levamisole (Vector Laboratories, Burlingame, California, USA) was added to quench endogenous alkaline phosphatase. Slides were counterstained lightly with haematoxylin and coverslipped for a permanent record.

Immunohistochemistry. Unstained sections were heated at 60°C for 15 min and deparaffinized in Hemo-De (PMP Medical Industries) for 20

Table 1.   *REV strains used in this study*

Sample	GenBank accession number	Genome size (base pairs)	Vírus description
REV-A	NC_006934	8295	Reticuloendotheliosis virus
REV-T	K00555 X02759	1923	Reticuloendotheliosis virus, proviral, oncogene—v-rel
REV-APC 566	DQ 387450	8286	Reticuloendotheliosis virus
REV-RU	NA	NA	Reticuloendotheliosis virus[a]
REV-397 A	NA	NA	Reticuloendotheliosis virus[b]

NA, not available. [a]Isolated from a duck and provided by Dr K. A. Schat (see Li et al., 1983). [b]Isolated from frozen turkey tissue (see Crespo et al., 2002).

Table 2. *Samples of the archived formalin-fixed and paraffin-embedded tissues from natural and experimental reticuloendotheliosis cases*

Sample (blocks)	Origin	Species	Case	Age	Fixation[a]
7052 (1)	Dr Fadly, US Department of Agriculture	Chicken	Experimentally inoculated REV-SNV at hatch	140 days	2 days
7079 (2)	Dr Fadly	Chicken	Experimentally inoculated REV-SNV at hatch	179 days	2 days
7100 (2)	Dr Fadly	Chicken	Experimentally inoculated REV-SNV at hatch	126 days	2 days
7116 (1)	Dr Fadly	Chicken	Experimentally inoculated REV-SNV at hatch	57 days	2 days
R06-491 (1)	Dr Zavala, University of Georgia	Quail	Experimentally inoculated REV-APC-566	20 weeks	1 year
R06-492 (3)	Dr Zavala	Grouse (Attwater's prairie chicken)	Natural REV detected by PCR and virus isolation	3 years, 10 months	1 year
R06-493 (6)	Dr Zavala	SPF Turkey	Experimentally inoculated REV-APC-566	9 weeks	1 year
A-85746(2)	Dr Hafner, US Department of Agriculture	Turkey	NA	19 weeks	3 days
F00-283 (8)	Dr Prasad, University of California—Davis	Turkey	Natural REV isolated and detected by PCR	16 weeks	2 days
F00-699 (17)	Dr Prasad	Turkey	Natural REV isolated and detected by PCR	35 weeks	2 days

NA, not available. [a]10% formalin fixation time.

min. The slides were dried at 37°C for 20 min, and the sections were encircled by hydrophobic pen. Antigen retrieval was achieved by microwaving at full power in citrate buffer for 10 min. The sections were blocked with casein solution. A commercially available polyclonal avian reticuloendotheliosis antiserum (Charles River Laboratories, Wilmington, Massachusetts, USA) was used as primary antibody at a concentration of 1:5000 in PBS, and incubated at 4°C overnight. A secondary biotinylated anti-chicken antiserum (Vector) at a concentration of 1:5000 in PBS was applied, and the slides were incubated in a humid chamber at 37°C for 1 h. The Vectastain avidin–biotin complex–alkaline phosphatase (ABC-AP) kit (Vector) was used as the detection system, followed by a commercially available ABC-AP substrate kit (Vector Red), according to the manufacturer's instructions. Slides were then lightly counterstained with haematoxylin for 12 sec and coverslipped with Permount.

Statistical analysis. The level of agreement between IHC and ISH results was determined by the Kappa statistical test (Landis & Koch, 1977).

Table 3. *Primers and PCR product sizes used for generation of the riboprobes*

Probe	Sequence (5′ to 3′)	PCR product size (base pairs)
REV-gag sense 1	TGCGCAAGTTATGT GAGTCGGA	448[a]
REV-gag sense 2	AGGCGGCCCGTGCT CCCCTCAGC	259
REV-gag anti-sense 2	CCCGTTCCCCAGTTT CCCTAAGGGG	
REV-gag sense 3	GGAGGGCTCAATT CCTGGATG	151
REV-gag anti-sense 3	CCACTTCGGGTGT GGGGAGGGCTC	

[a]The product indicated for the primer "REV-gag Sense 1" (448 base pairs) was obtained amplifying with the primer "REV-gag Anti-Sense 2".

Results

No neoplastic transformation was observed in haematoxylin and eosin-stained sections from experimentally infected Japanese quail embryos. None of the infected embryos had any significant histological change in any of the organs examined.

Positive staining was observed in the sections of Japanese quail embryos infected with all REV strains used in this study (Table 1). The strongest signal was obtained with the longer anti-sense riboprobe (500 bases), whereas the smaller ribroprobes (259 and 151 bases) resulted in positive but less intense labelling (data not shown). Sense probes, did not result in any positive staining, thus confirming the specificity of the assay. Non-specific background staining was negligible.

The viral genome of all REV strains used in this study was detected by ISH mostly in the proventriculus (Figure 1). Strong positive staining was observed in both the epithelium and musculature of the proventriculus in the majority of the infected embryos (76%), indicating a viral tropism for the proventriculus (Table 4). In addition, strain REV-T was also detected in the endothelium of blood vessels in two out of five infected embryos. All strains studied were able to infect the embryos.

All infected embryos were also positive by IHC. PBS-inoculated Japanese quail embryos were all negative by IHC and non-specific background staining was negligible. Strong positive IHC staining was observed in the cytoplasm and nuclei of cells, particularly those in the proventriculus with a tissue distribution similar to the ISH staining, regardless of the REV strain inoculated (Figure 1 and Table 5). REV antigens were also detected in the heart of two embryos infected with different strains; in endothelial and muscle cells from two embryos infected with strain REV-T, and in chondrocytes of one embryo infected with strain REV-APC 566 (Table 5). Thus, these IHC results further confirm the susceptibility of Japanese quail embryos to REV infection.
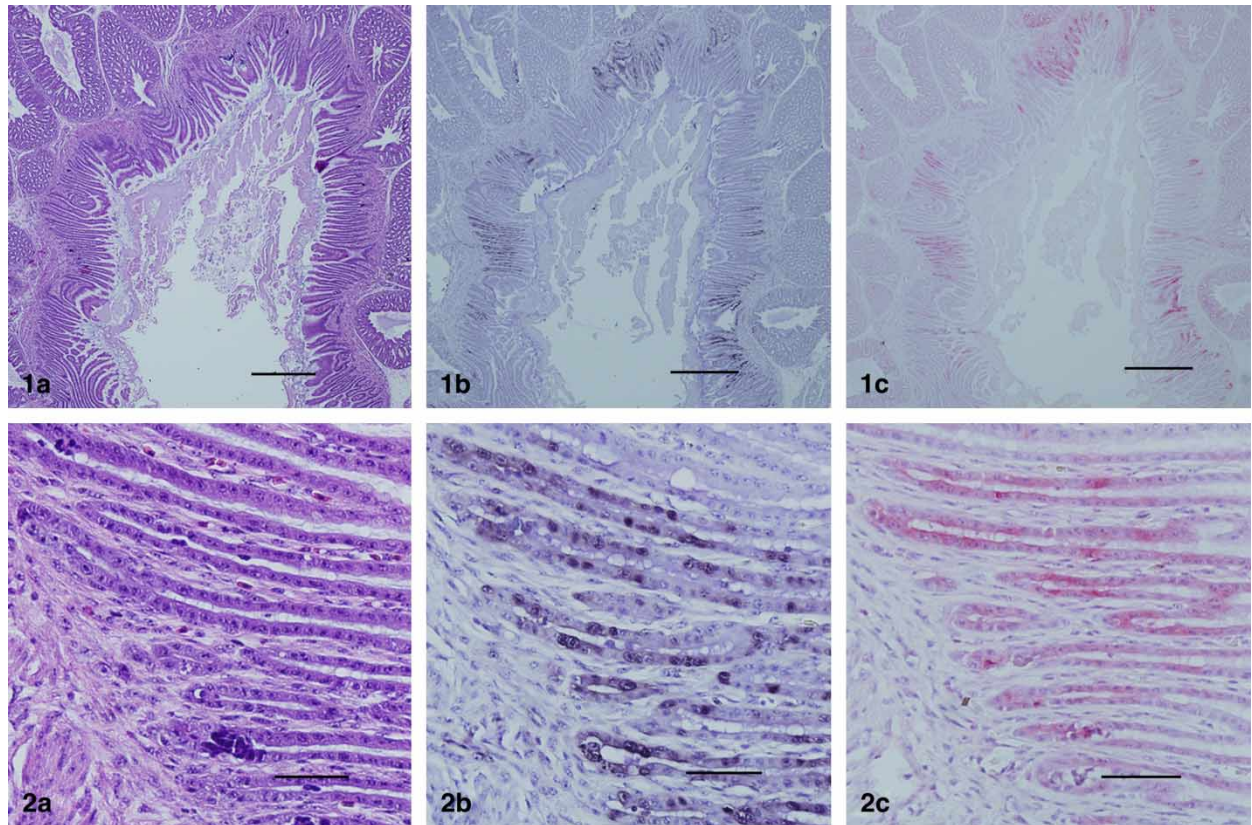
Figure 1. *Japanese quail embryos experimentally infected with REV strain RU. Proventriculus counterstained with haematoxylin and eosin (1a and 2a), with epithelial cells positive by ISH (1b and 2b) and by IHC (1c and 2c). Bar = 80 μm.*
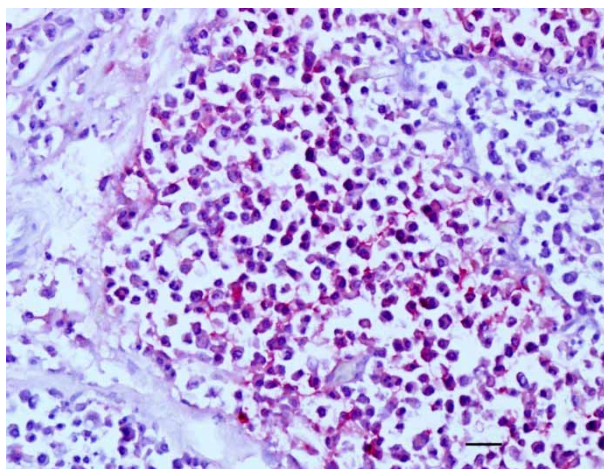
These IHC and ISH protocols were then applied to formalin-fixed and paraffin-embedded tissues derived from archived naturally occurring and experimentally induced reticuloendotheliosis tumours, totalling four chickens, four turkeys, one grouse (Attwater's Prairie chicken) and one quail (Table 2). All tumours consisted of relatively uniform populations of lymphoid cells with minimal stroma. The REV genome and REV antigens were detected in only one of the 10 birds. One of the

Figure 2. *Bursa of Fabricius, chicken, 140 days old, experimentally infected with REV strain SNV at hatching, with lymphoid neoplasia positive by immunohistochemistry. Bar = 60 μm.*

chickens experimentally inoculated with REV (strain REV-SNV) that had developed a lymphoma involving the bursa of Fabricius (Table 2, case 7052) was positive by both ISH and IHC (Figure 2).

When all of the results of organs that had positive staining in at least one animal were combined together, Kappa statistics indicated an almost perfect agreement between IHC and ISH results (Kappa index = 0.916; $P < 0.001$).

Discussion

Owing to the lack of suitable diagnostic approaches for detecting active REV infection in formalin-fixed paraffin-embedded tissues, we developed assays for the detection of REV antigens and genomic sequences, by IHC and ISH, respectively. These assays proved to be specific and resulted in detection of several different REV strains in experimentally infected Japanese quail embryos. Quail embryos as a model for infection was established by Barbosa *et al.* (2007b), who showed that the oncogenic potential in quail is the same as in other commercial poultry species, such as turkeys and chickens. Compared with chickens, the smaller size of quails shortens the development time and enables more birds per unit area. However, both IHC and ISH demonstrated a low sensitivity of detection of REV in well-developed neoplasia as demonstrated by the low frequency of staining in the tested natural and experimental reticuloendotheliosis cases. The negative results in some of the samples could be due to the lack of an active infection with no RNA being transcribed and

Table 4.   *Tissues positive for REV by ISH in each strain used[a]*

Tissue	Strains (n = 5)[b]				
	REV-A	REV-T	REV-APC 566	REV-RU	REV-397 A
Proventriculus (epithelium)	4	4	5	3	3
Proventriculus (musculature)	4	3	4	3	2
Blood vessel (endothelium)	0	2	0	0	0

[a]Only positive stained tissues were included in the table, although several tissues were examined in serial sections. [b]n = 5 for each strain.

Table 5.   *Tissues positive for REV by IHC in each strains used[a]*

Tissue	Strain (n = 5)[b]				
	REV-A	REV-T	REV-APC 566	REV-RU	REV-397 A
Proventriculus (epithelium)	4	4	5	3	3
Proventriculus (musculature)	4	3	4	3	2
Heart	0	1 (endocardium)	0	1 (endocardium and myocardium)	0
Blood vessel (endothelium)	0	2	0	0	0
Musculature	0	2	0	0	0
Cartilage (chondrocyte)	0	0	1	0	0

[a]Only positive stained tissues were included in the table, although several tissues were examined in serial sections. [b]n = 5 for each strain.

therefore no translation and no antigenic viral proteins present in the infected tissue (Swift *et al.*, 1987; Nuovo, 2007). This notion of lack of active infection in chronic cases is supported by the observation that REV-infected lymphoid cells, transformed *in vitro*, can infect but not cause lethal reticuloendotheliosis in compatible birds (Lewis *et al.*, 1981). In addition, archived paraffin-embedded tissues from different laboratories usually undergo variable processing protocols, which in some circumstances may favour RNA degradation and deterioration of epitopes that impair the sensitivity of ISH and IHC, respectively (Brown, 1998, Ramos-Vara, 2005).

The assays developed in this study have a great potential as diagnostic tools in cases of active REV infections that may result in immunosuppression, runting syndromes, high mortality, or lymphomas (Witter *et al.*, 1986; Witter & Fadly, 2003). In the present study, IHC and ISH yielded highly similar results, with both assays able to detect infected tissues from Japanese quail embryos inoculated with different REV strains.

REV genomic sequences and antigens were detected by ISH and IHC in some organs of the Japanese quail embryos, but were most consistently detected in the proventriculus (Tables 4 and 5). Strong positive staining was observed in both the epithelium and musculature of this organ in most embryos (76%) infected with different REV strains, a finding that indicates a viral tropism for the proventriculus. Interestingly, proventriculitis has been previously described in association with REV infection, although these previous studies were unable to establish a cause-and-effect relationship between viral infection and the lesions in the proventriculus (Jackson *et al.*, 1977; Bagust *et al.*, 1979). However, there were no significant gross or histological lesions observed in the proventriculi of this study; it is unclear whether this lack of response was due to the route of inoculation, species

infected, or to the age of the embryos. This strong viral tropism for the proventriculus is in marked contrast to some other lymphoma-inducing retroviruses such as ALV subgroup J, which has a much broader tissue distribution (Stedman *et al.*, 2001; Williams *et al.*, 2004; Hafner *et al.*, 2007).

There are other diagnostic techniques for REV (Davidson *et al.*, 1995; Crespo *et al.*, 2002). PCR is the most sensitive in detecting tissues infected with REV. Viral isolation and indirect immunofluorescence are also useful, but both of these require fresh or frozen materials. All three of these techniques lack the ability to gain contextual information; that is, to see evidence of the virus within the background of any pathologic changes. ISH and IHC allow for simultaneous interpretations of cellular/tissue tropism, pathologic damage, and presence of virus. In addition, IHC and ISH are suitable for formalin-fixed and paraffin-embedded tissue samples, which are often the tissues available in diagnostic laboratories.

IHC has been often used for detection of Avian leukosis virus (ALV) and Marek's Disease virus (MDV) in tissues (Gharaibeh *et al.*, 2001), but not for REV (Witter & Fadly, 2003). To the best of our knowledge this is the first study aiming to develop IHC and ISH assays for detection of active REV infection in tissues. These assays have a great potential for diagnostic purposes as alternative methods for identifying REV in tissues.

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